A2 (high affinity), are potent at inhibiting Na+ dependent phosphate co-transport and vitamin D metabolism in a human renal cell line (CL8). The high affinity fraction, the human renal cell line (CL8), and the conditions used for assay are described in Rowe *et al* 1996. A further suitable known renal cell line for this assay is the OK cell line deposited as ECACC 91021202.

Cation exchange Chromatography using HiTrap SP cation exchange 1 ml column (Code No 17-1151-01; Pharmacia):

1. The lyophilized protein was then re-dissolved in 0.05M ammonium acetate pH 5 and the applied to an equilibrated 1 ml HiTrap SP sepharose cation exchange column.
2. The column was equilibrated prior to sample addition by washing with water, and then 5 volumes of start buffer (0.02 M ammonium acetate pH 5).
3. Sample was eluted using the following protocol;

Num	Time min	Flow rate ml /min	%NH^4 acetate pH5	% NH^4 acetate/ 0.5M NaCl pH 5
1		0.5	100	0
2	15	0.5	25	75
3	20	0.5	0	100
4	25	0.5	0	100
5	35	0.5	100	0
6	50	0.5	100	0

A Single sharp peak was obtained, and the sample was then dialyzed against 0.05M acetic acid and lyophilized; see Figure 2.

After resuspending in 10 mM phosphate buffer pH 7.2 20 µl, aliquots were resuspended in SDS-PAGE sample buffer (to a final concentration = 125mM TRIS-HCL pH6; 2.5% glycerol; 0.5% w/v SDS: 5% β-mercaptoethanol; 0.01% bromophenol blue), boiled (5 mins), cooled and then run on an SDS PAGE gel 12.5% (see chromatogram), and a double band of 55 kD was resolved (see Rowe *et al* 1996). Both the Concanavalin A and cation bands also have an aggregated form. All fractions including the tumor conditioned media were potent at inhibiting

Na^+ dependent phosphate co-transport in a human renal cell line (1/1000 diln), and also altered vitamin D metabolism. For a full description of the methods used to measure phosphate transport and vitamin D metabolism see Rowe *et al* 1996. All purification modalities were carried out on a waters HPLC/FPLC system programmed by computer-millennium software. The most active fraction was the concanavalin A1 fraction from OHO tumor. Anti pre-operation antisera was used to screen the immobilized purified fraction. The fraction is also potent at inhibiting NaPi, and affects vitamin D metabolism in a human renal cell line (CL8).

Example 2: Screening of tumor conditioned-medium (TCM), and purified fractions with pre/post- operation antisera: plus glycoprotein screen

Pre-operation and post-operation antisera from a patient has been described previously in Rowe *et al* 1996. Only pre-operation antisera detected the purified fractions and hormone in TCM in which Western and glycoprotein detection of TCM and purified fractions was achieved using enhanced chemiluminescence. Protein markers were biotinylated, and tagged with streptavidin peroxidase conjugate. The arrows show the aggregate and active glycoprotein. Post-operation antisera and rabbit pre-immune sera did not detect any of the fractions. Also, only those tumors secreting phosphaturic factor were positive. Media and skin controls were negative. A distinct feature of the Con A1, Con A2 and CA1 samples was their potent ability to inhibit NaPi, and alter vitamin D metabolism in a human renal cell line (CL8). All the purified fractions have a tendency to aggregate into a lower mobility form on SDS-PAGE. Also, the purified fractions and TCM active fractions are heavily glycosylated. The extent of glycosylation was confirmed by periodate oxidation of immobilized proteins on PVDF membranes followed by biotinylation of carbohydrate moieties. These were then screened with streptavidin conjugated to horse radish peroxidase and enhanced chemiluminescence. The active form (inhibits NaPi etc.), is associated with the 58 to 60 kDa fraction. An additional and powerful way of purifying the protein to homogeneity is the use of a neutral pH 7 SDS-PAGE system using a 4-12% Bis-Tris Gel with MOPS running buffer. Pre-caste gels can be purchased from Novex.

Example 3: SDS-PAGE at neutral pH using 4-12% polyacrylamide gradient and Bis-Tris gel with MOPS running buffer (Nu-PAGE system from NOVEX): Reduced mobility of hormone

On this system a fraction of the glycosylated hormone has a reduced mobility, and runs at ~200 kDa. The lower molecular weight form is also visible at 58/60 kDa. Appearance of the ~200kDa protein may be due to the isoelectric point of the protein (different charge at neutral pH), and the interaction of carbohydrate moiety with the gel matrix. Also, increased efficiency of electro-blotting of high molecular weight components occurs due to the low % acrylamide (4-12% gradient), at the top of the gradient gel. Running fractions through this system increases the purity and homogeneity of the molecule. A Western blot using this system and including the following samples (pre-operation antiserum was used to screen the blots using enhanced chemiluminescence detection): 1. protein markers; 2. intracranial tumor cell line OHO; 3. cells from sub-dura adjacent to tumor; 4. cells from dura adjacent to sub-dura; 5. HTB6 cell line; 6. Saos-2 cell line; 7. defined medium control; 8. Skin fibroblast control; 9. Linear sebaceous naevus polyp tumor demonstrated that Naevus polyp tumor showed a specific phosphaturic band at ~200kDa on SDS-PAGE Neutral gels.

Example 4: Cloning and Sequencing of Phosphatonin

1. Library construction:

A tumor derived from a patient described in an earlier publication (BD, Rowe et al., 1996), was sectioned and mRNA extracted using standard techniques. The mRNA was copied using reverse transcriptase to generate a cDNA population that was then subsequently subcloned into a bacteriophage vector λ-ZAP II uni (vector purchased from Stratagene Ltd., Unit 140, Cambridge Science Park, Milton Road, Cambridge, CB4 4GF United Kingdom). The cloning was uni-directional and the 5' end of the gene was adjacent to the T3 promoter and abutted an EcoRI site. The 3' end of the cDNA's abutted an Xho-1 site upstream of a bacterial T7 promoter. Briefly, resected tumour from patient BD was cut into 1 mm blocks and poly A+ RNA extracted directly using Streptavidin-Magnesphere paramagnetic particle technology (PolyATract® system Promega). The purified mRNA was then used to generate a cDNA template using the cDNA synthesis kit from Stratagene. Linker